CHARACTERIZATION OF A PETUNIA STRAIN OF TURNIP VEIN-CLEARING VIRUS

S. Sabanadzovic[1], N. Abou Ghanem-Sabanadzovic[2], A. Henn[1] and A. Lawrence[3]

[1] *Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA*
[2] *Department of Plant Pathology, University of California, Davis, CA 95616, USA*
[3] *Electron Microscope Center, Mississippi State University, Mississippi State, MS 39762, USA*

SUMMARY

Virus-like symptoms were observed in February 2005 on Double Wave™ petunias (*Petunia x hybrida* Hort. Vilm.-Andr.) grown in a greenhouse in northeastern Mississippi. Electron microscope observations of leaf dips from symptomatic plants showed the presence of rod-shaped virus-like particles. Infected petunia samples reacted weakly with an immunostrip test to *Tobacco mosaic virus* (TMV), but not in ELISA using a polyclonal anti-TMV-c commercial kit. The dsRNA patterns from four infected petunias were identical and resembled those of tobamoviruses although they differed slightly in size from the replicative forms of TMV extracted from infected tobacco plants. Purified dsRNAs were used for random-primer cloning of the viral genome. A partial sequence of the viral 3' end showed that the virus is a strain of *Turnip vein-clearing virus* (TVCV, reported also as *Tobacco mosaic virus*-crucifer strain) sharing 96% common nucleotides and 98% identical amino acids. To the best of our knowledge, TVCV has not been reported previously either from petunias or from Mississippi in general.

Key words: petunia, tobamovirus, *Turnip vein-clearing virus*, dsRNA, antibodies, sequencing, RT-PCR.

INTRODUCTION

Viruses belonging to several genera have been reported to cause considerable economic losses to the petunia industry (*Petunia x hybrida* Hort. Vilm.-Andr.; *Petunia hybrida* Vilm.) (Lesemann, 1996; Spence *et al*., 1996; Sanchez-Cuevas and Nameth, 1996, 2002; Feldhoff *et al*., 1998; Alexandre *et al*., 2000; Liu *et al*., 2003). In addition to the intrinsic susceptibility of petunias, the risk of viral infections was further increased by the introduction of vegetatively propagated petunia hybrids in the early 1990's, which led to serious disease outbreaks.

Virus-like symptoms consisting of poor growth, leaf mottling and distortion, as well as discoloration/deformation of the petals were observed on potted Double Wave™ petunias in a greenhouse near Starkville, Mississippi in February 2005. Visual estimates indicated that *ca.* 40-45% of the entire petunia stock was affected by the disease.

Four representative samples were taken from the diseased plant lot for laboratory analyses aimed at identification of the causal agent. The results of this study are presented herein.

MATERIALS AND METHODS

Virus source and experimental host range. Virus sources were four infected double-flower petunia plants showing stunting, mottling and slight petal discoloration. Leaf extracts from each infected plant were transferred separately onto celite-dusted leaves of five herbaceous species of the families *Solanaceae* and *Chenopodiaceae* and three different petunia cultivars (Red Madness, Plum Madness and Pink Madness). Plants were kept in a greenhouse at 20-22ºC and observed daily for symptom expression for 4 weeks.

In addition, a common strain of *Tobacco mosaic virus* (TMV), isolated from tomato plants in Mississippi (S. Sabanadzovic, unpublished) was propagated in tobacco plants, and used for comparisons in some phases of this work.

Electron microscopy. Leaf dip and/or partially purified virus preparations were mounted in 2% aqueous uranyl acetate prior to observation with a JEOL JEM 100CXII electron microscope. Virus was purified by polyethylene glycol (PEG) precipitation and further concentrated by alternate high- and low-speed centrifugation. For cytopathological observations, infected petunia leaves were excised and processed according to Martelli and Russo (1984) with slight modifications. Tissue was fixed with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, post-fixed in 2% osmium tetroxide then

Corresponding author: S. Sabanadzovic
Fax: +01.662.325-8837
E-mail: ss501@msstate.edu

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dehydrated in a graded ethanol series prior to embedding in Spurr's resin. Ultra-thin sections were cut on a Reichert-JUNG UltracutE microtome and stained with uranyl- and lead citrates before observations.

Serology. Immunostrip and DAS-ELISA tests were performed using commercial kits (Agdia, USA) to TMV, TMV-c, and *Tomato mosaic virus* (ToMV). For Western blot analyses, total proteins extracted from the original petunias, TMV-infected tobacco plants and healthy controls were dissociated, electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) discontinuous system (Laemmli, 1970) and blotted onto a nitrocellulose membrane with a semi-dry Trans-blot apparatus (Bio-Rad, USA) as described by Hu *et al.* (1990). After overnight blocking in TBS buffer (0.02 M Tris, 0.5 M NaCl) containing 5% nonfat milk powder, 1% bovine serum albumin (BSA) and 0.05% Tween-20, the membranes were exposed for 2 h at 37°C to anti-TVCV polyclonal antiserum (diluted 1:50 in the same buffer) and commercial anti-TMV antibodies (Agdia, USA) (diluted 1:100), washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgGs diluted 1:2000. Reactions were visualized by soaking membranes in a solution of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in substrate buffer (0.1 M Tris, 0.1 M NaCl and 5 mM $MgC1_2$, pH 9.5) (Hsu and Lawson, 1991).

DsRNA analysis and cloning. Double-stranded RNAs (dsRNA) were isolated from diseased samples using phenol-chloroform extraction and CF-11 column chromatography (Valverde *et al*., 1986). Healthy petunia plants and TMV-infected tobacco plants were used as controls. The extracted dsRNAs were analyzed by 6% PAGE in tris-acetate (TAE) buffer and visualized by staining with silver nitrate.

Purified dsRNA preparations were reverse transcribed with random primers and amplified by Degenerate Oligonucleotide Primers (DOP) using a commercial kit (Roche Applied Science, USA). Generated PCR products were cloned into pGEM-T Easy plasmid (Promega, USA) and the resulting recombinant plasmids were transferred in *Escherichia coli* Top 10 competent cells. DNAs of plasmids from selected colonies were sequenced at the DNA Sequencing Facility, Life Science Biotechnology Institute of Mississippi State University. Sequences between adjacent clones were generated by RT-PCR using virus-specific primers. Sequence data were analyzed by Lasergene software and compared with sequences available in the NCBI/GenBank using BLAST on-line resource (Altschul *et al*., 1997).

RT-PCR. A set of virus-specific primers was designed in order to develop a reliable RT-PCR method for virus detection. Primers were designed to amplify a 720 nt portion of the viral genome and were applied on reverse-transcribed total RNAs extracted from infected and healthy petunia samples using Qiagen Plant RNeasy kit (Qiagen, USA), according to the manufacturer's instructions. Moreover, a set of "universal" primers for the genus *Tobamovirus* (Dovas *et al*., 2004) was used to check for the possible presence of additional tobamoviruses in infected plants. PCR products from all four original petunia samples were cloned as described, and 10 randomly-chosen clones per sample were sequenced from each sample (total of 40 clones).

Koch's postulates. To prove involvement of the virus in the disease, partially purified virus preparations obtained from infected *Nicotiana* plants after two cycles of sucrose density gradients were mechanically inoculated onto six healthy Double Wave petunia plants kindly provided by Ball Horticultural Company (West Chicago, USA). Plants were kept under controlled temperature and light conditions and observed for symptoms for 45 days. Three weeks post-inoculation tissues from these plants were used for re-isolation of the virus by mechanical inoculation. Three mock-inoculated petunia specimens were kept under the same conditions and used as controls.

RESULTS AND DISCUSSION

All plant species used in mechanical transmission were susceptible to infection by the petunia virus. Responses varied according to the host and ranged from local lesions 2-3 days post-inoculation (*Chenopodium amaranticolor* and *Nicotiana glutinosa*) to systemic mosaic/mottling approximately one week after inoculation (*N. benthamiana*, *N. rustica*, *N. tabacum* cv. Turkish). Inoculated petunia cultivars exhibited a variety of symptoms including transient sepal necrosis (Fig. 1A), colour break of the petals, ring spots and line patterns (Fig.1B).

Electron microscope observations of leaf extracts revealed the presence of rigid rods resembling tobamovirus particles in the four tested petunias (Fig. 1C). Similar particles were observed in artificially inoculated hosts. The cytopathology of naturally infected petunia cells conformed to that induced by TMV and other tobamoviruses in host plants (see among others Esau and Cronshow, 1967; Esau, 1968). Virus particles were plentiful, forming large layered aggregates in the cytoplasm of infected petunias. They often associated with plasmalemma and other membranes (Fig. 1D).

Infected petunia samples reacted positively in immunostrip and DAS-ELISA tests with TMV antibodies (Agdia cat. No STX 57400 and PSA 57400), respectively. However, the intensity of reaction was much weaker than in the case of the TMV-positive control. In addition, the

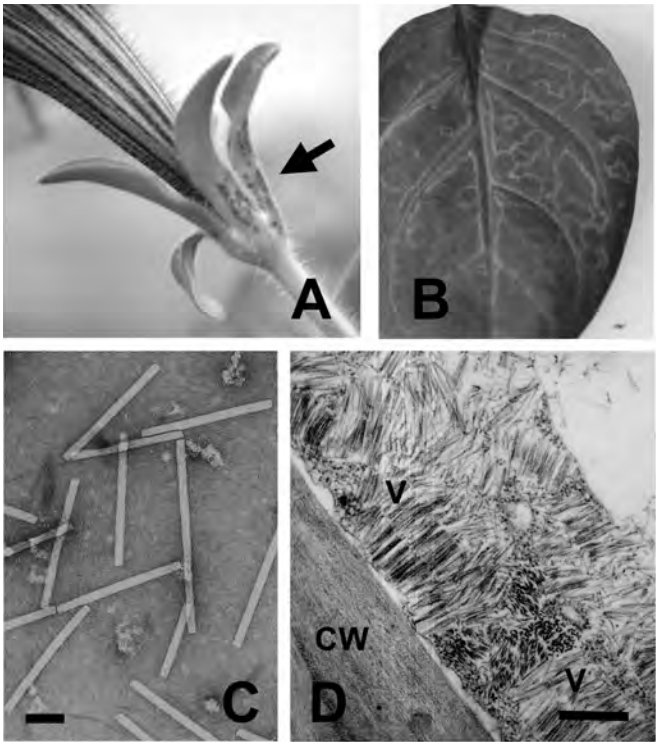

Fig. 1. Artificially inoculated petunias cv. Plum Madness showing transient sepal necrosis (panel **A**, arrows) and arabesque mosaic (panel **B**). **C.** Negatively stained virus particles observed in partially purified leaf extracts from originally infected petunias. Bar represents 100 nm. **D.** Layered aggregates of virions (V) present in the parenchyma cells of infected petunias (CW - cell wall, Vac- vacuole). Bar represents 500 nm.

same preparations did not react with an anti-TMV-c commercial kit (Agdia, cat. No PSA 57500) (Table 1). This provided evidence that the petunia virus was serologically related to but distinct from TMV. As it was reported that the TMV kit detects a variety of tobamoviruses including a common strain of this virus (TMV-c), *Tomato mosaic virus* (ToMV) and *Sunn hemp mosaic virus* (SHMV) (http://www.agdia.com/cgi_bin/catalog.cgi?SRA+57400%2F0096), the petunia samples were also tested with the anti-ToMV kit (Agdia cat. No PSA 35400). A weak reaction with ToMV antibodies was observed (Table 1), confirming that the petunias virus is a tobamovirus serologically related, but distinct from either TMV or ToMV.

All originally infected samples contained a range of identical, high molecular weight dsRNA bands when analyzed by PAGE. When compared, the dsRNA profiles from petunias differed slightly in migration rate from the viral replicative forms extracted from TMV-infected tobacco plants (Fig. 2A). In particular, dsRNA bands corresponding to the replicative forms of genomic RNA (gRNA) and subgenomic RNA-1 (sgRNA-1) of the petunia virus had a smaller size than the corresponding TMV dsRNAs. In contrast, the sgRNA-2 of the petunia virus, coding for the viral coat protein, had a larger size than the equivalent TMV molecule.

Table 1. Optical density readings at 405 nm after 30 min in enzyme immunoassays. Values represent an average of four readings.

	Antiserum		
	TMV	TMV-c	ToMV
TMV[(a)]	2.920	0.959	1.362
TMV-c[(a)]	2.860	1.292	2.328
ToMV[(a)]	2.556	0.235	2.759
Infected petunias (original)	0.376	0.071	0.339
Healthy control A (petunia)	0.076	0.072	0.085
Healthy control B (tobacco)	0.070	0.065	0.079

* Positive controls from Agdia Inc.

The sequence of 3,309 nt, representing ca. 55% of the whole genome (Genbank accession No. EU413669) comprises the C-terminal part of the viral replicase gene (partial viral helicase and complete motifs of RNA-dependent RNA polymerase), complete viral movement and capsid proteins as well as the entire 3' non-coding region. Comparative gene-by-gene sequence analyses revealed that the petunia virus had high sequence homology with *Turnip vein-clearing virus* (TVCV) (ca. 98-99% in amino acid sequences) (Lartey *et al*., 1995) and to a lesser extent (*ca.* 45 to 60%) with other tobamoviruses.

Western blots with polyclonal antibodies to TVCV and TMV confirmed the serological relationship between the two viruses, for both antisera cross-reacted with the heterologous antigen (Fig. 2B), but not with healthy controls.

The set of primers designed in this work proved reliable and specific for RT-PCR detection of TVCV. An amplicon of the predicted size of 720 bp was generated from all tested TVCV-infected petunias (Fig. 2C). Moreover, all 40 clones generated by universal tobamovirus primers contained only TVCV sequences.

All the above data clearly indicate that the virus isolated from diseased petunias is a strain of TVCV, a tobamovirus originally reported from turnip plants from Oklahoma and later recovered from different crucifers (Lartey *et al*., 1993, 1995; Dorokhov *et al*., 1994; Yamanaka *et al.*, 1998).

Inoculation of purified TVCV preparation to a new set of healthy Double Wave^{TM} petunias reproduced the original symptoms. The virus was re-isolated by mechanical inoculation to *N. tabacum* cv. Turkish (not shown), and its identity confirmed by virus-specific RT-PCR.

In conclusion, considering that by electron microscopy, mechanical transmission, dsRNA extraction and RT-PCR no virus other than TVCV was detected in infected petunias, its involvement in the etiology of the disease was hypothesized and confirmed by fulfilling

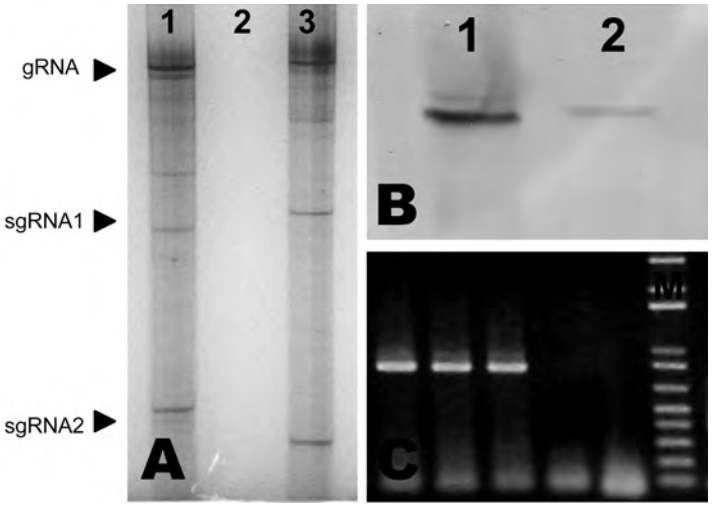

Fig. 2. A. Electrophoretic patterns of dsRNAs extracted from TVCV-infected petunias (lane 1), healthy control (lane 2) and TMV-infected *Nicotiana tabacum* (lane 3). gRNA: full-genomic size replicative forms, sgRNA: replicative forms of sub-genomic RNAs 1 and 2. **B.** Western blot using anti-TVCV serum. Serological correlation is evident even though capsid proteins of TVCV-petunia strain (lane 1) and TMV (lane 2) have a slightly different migration rate. **C.** Ethidium bromide stained gel of PCR products generated with TVCV-specific primers. Products of the expected size were amplified only from infected petunias (lanes 1-3). No amplicons were obtained fom TMV-infected tobacco (lane 4) and healthy petunia plant (negative control). DNA ladder is in lane M.

Koch's postulates. It was also shown that TVCV infections induce damage not only to double-wave type but also to single flower petunias.

TMV and ToMV are the only tobamoviruses reported from petunia (Lesemann, 1996; Alexandre *et al*., 2000; Spence *et al*., 2001). The positive identification of TVCV reported in this paper suggests that this virus could be the unidentified TMV-like pathogen frequently detected in single or double flower petunias from Ohio (Sanchez-Cuevas and Nameth, 2002) and by Agdia's Testing Services (Anonymous, 2005). If true, the overall importance of this virus in petunias may be much greater than an accidental infection, thus requiring particular attention.

To the best of our knowledge, this seems to be the first report of TVCV in petunias and solanaceous plants in general, as well as the first record of this virus in Mississippi and southern United States.

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REFERENCES

Anonymous, 2005. New "Tobacco mosaic" problem on petunias. Agdia Testing Service Pathogen leaflet (http://www.agdia.com/testing/pathogen.leaflets/tobamo.petunia).

Alexandre M.A.V., Soares R.M., Rivas E.B., Duarte L.M.L., Chagas C.M., Saunal H., Van Regenmortel M.H.V., Richtzehain L.J., 2000. Characterization of a strain of Tobacco mosaic virus from *Petunia*. *Journal of Phytopathology* **148**: 601-607.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Dorokhov L.Y., Ivanov P.A., Novikov V.K., Agranovsky A.A., Morozov S.Y., Efimov V.A., Casper R., Atabekov J.G., 1994. Complete nucleotide sequence and genome organization of a tobamovirus infecting cruciferae plants. *FEBS Letters* **350**:5-8.

Dovas C.I., Efthimiou K., Katis N.I., 2004. Generic detection and differentiation of tobamoviruses by a spot nested RT-PCR-RFLP using dI-containing primers along with homologous dG-containing primers. *Journal of Virological Methods* **117**: 137-144.

Esau K., 1968. Viruses in Plant Hosts. Form, Distribution and Pathologic Effects. The University of Wisconsin Press, Madison WI, USA.

Esau K., Cronshaw J., 1967. Relation of tobacco mosaic virus to the host cell. *Journal of Cell Biology* **33**: 665-678.

Feldhoff A., Wetzel T., Peters D., Kellner R., Krczal G.,1998. Characterization of petunia flower mottle virus (PetFMV), a new potyvirus infecting *Petunia x hybrida*. *Archives of Virology* **143**: 475-488.

Hsu H.T., Lawson R.H., 1991. Direct tissue blotting for detection of tomato spotted wilt virus in impatiens. *Plant Disease* **75**: 292-295.

Hu J.S., Gonsalves D., Teliz D., 1990. Characterization of closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology* **128**: 1-14.

Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacterial phage T4. *Nature* **227**: 680-685.

Lartey R.T., Hartson D.S., Pennington R.E., Sherwood J.L., Melcher U., 1993. Occurrence of vein-clearing tobamovirus in turnip. *Plant Disease* **77**: 21-24.

Lartey R.T., Voss T.C., Melcher U., 1995. Completion of a cDNA sequence from a tobamovirus pathogenic to crucifers. *Gene* **166**: 331-332.

Lesemann D.-E., 1996. Viruses recently detected in vegetatively propagated petunia. *Acta Horticulturae* **432**: 88-94.

Liu H., Sears J.L., Bandla M., Harness A.M., Kulemeka B., 2003. First report of Calibrachoa mottle virus (CbMV) infecting petunia. *Plant Disease* **87**: 1538.

Martelli G.P., Russo M., 1984. Use of thin sectioning for the visualization and identification of plant viruses. *Methods in Virology* **8**: 143-224.

Sanchez-Cuevas M.C., Nameth S.G.P., 1996. Viruses associated with mosaic and white leaf in double petunia. *Phytopathology* **86**: S652.

Sanchez-Cuevas M.C., Nameth S.G.P., 2002. Virus-associated diseases of double petunia: Frequency and distribution in Ohio greenhouses. *HortScience* **37**: 543-546.

Spence N.J., Barker I., O'Neill T., Bennison J., Wright D., 1996. Viruses in trailing petunias. *HDC Fact Sheet* No 04/96.

Spence N.J., Sealy I., Mills P.R., Foster G.D., 2001. Characterization of a tobamovirus from trailing petunias. *European Journal of Plant Pathology* **107**: 633-638.

Valverde R.A., Dodds J.A., Heick J.A., 1986. Double-stranded ribonucleic acid from plants infected with viruses having elongated particles and undivided genomes. *Phytopathology* **76**: 459-465.

Yamanaka T., Komatani H., Takahashi M., Meshi T., Naito S., Ishikawa M., Ohno T., 1998. Complete nucleotide sequence of the genomic RNA of Tobacco mosaic virus strain Cg. *Virus Genes* **16**: 173-176.

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